Evaluation of a Wastewater Discharge using Vitellogenin Gene Expression and Plasma Protein Levels in Male Fathead Minnows

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ABSTRACT

- Liver vitellogenin gene expression and plasma vitellogenin protein presence, indicators of exposure of fish to estrogens, were measured in male fathead minnows (*Pimephales promelas*) caged at two locations in a constructed wetland below a sewage treatment plant effluent outfall in Pecan Creek, TX. Control fish were held in activated charcoal-dechlorinated tap water in the laboratory at similar densities.
- Adult fish from the USEPA Aquatic Research Facility were shipped to the University of North Texas and held for a 2-day acclimation period before deployment. Ten fish were collected at 0, 1, 2, 4, 7, 14 and 21 days.
- At these intervals male secondary sex characteristics were scored; hepatic and gonadal somatic indices (HSI, GSI) were calculated; livers analyzed for vitellogenin gene expression; blood analyzed for vitellogenin protein; and hematocrit and fish condition factor were determined.
- Equilenin, estradiol, estrone and ethynylestradiol were analyzed in water collected at the two exposure locations on days 1, 7 and 21.
- Plasma vitellogenin protein was detected in fish at site 1 within 2 days of exposure and peaked within 7 days.
- Liver vitellogenin gene expression followed a similar trend.
- HSI at site 1 was significantly elevated by the end of the exposure period, but GSI was not simultaneously decreased.
- Results indicate that liver vitellogenin gene expression and plasma protein were higher in male fish caged closest to the wastewater discharge than in laboratory controls.
- Additionally, at the site closest to the effluent (site 1), the secondary sex characteristics fatpad thickness and number of breeding tubercles decreased with exposure time and were significantly decreased toward the end of the exposure period.

INTRODUCTION

There is a growing body of scientific evidence describing adverse biological changes resulting from exposure of laboratory animals, wildlife, and humans to a diverse class of chemical stressors collectively referred to as endocrine disrupting compounds (EDCs) [1-4]. Studies reported in the literature provide evidence both for and against the role of environmentally persistent chemicals in endocrine disruption [5-7]. The degrees to which human and wildlife populations are exposed to EDCs and the reduction of uncertainties in the assessment of risk to both humans and wildlife from exposure to EDCs have been a major focus of investigation. The U.S. Environmental Protection Agency Office of Research and Development and other scientific stakeholders [8] proposed to develop exposure screening tools and indicators for endocrine disrupting compounds.

We have developed a highly specific, molecular indicator that is diagnostic for exposure of aquatic organisms to environmentally relevant concentrations of 17α-ethynylestradiol (EE2), a potent EDC identified in wastewater effluent [9]. Environmentally persistent chemicals that functionally mimic natural estrogen are ubiquitous in surface waters and have been shown to affect the reproductive health of species living in these habitats [10-12]. Our exposure indicator allows for immediate measurement of cumulative estrogenic potential in aquatic systems and assesses the bioavailability of known estrogenic compounds, both alone and in complex mixtures.

The objective of this study was to look at the relationship and changes in Male Fathead Minnow liver vitellogenin (Vg) mRNA, Vg protein in plasma, Secondary Sex Characteristics (SSC), Gonato-Somatic Index (GSI), and Hepato-Somatic Index (HSI) over time during an in-situ exposure to a wastewater treatment plant effluent for 3 weeks.

METHODS

Adult Fish Used for Deployment in Wastewater.

- Two hundred 8 - 11 month old adult male fathead minnows (*Pimephales promelas*) were obtained from our laboratory cultures and shipped overnight (11/27/00) to the University of North Texas. All fish were at a stage of maturity such that male secondary sexual characteristics (tubercles, spongy fat pad on head, kark color banding) were discernible. This permitted males to be readily distinguished and separated from females. All efforts were made to segregate male fish from females during holding in order to reduce any potential sex-related influences.
- Prior to exposure, adult male fathead minnows were kept in 72 liter aquaria with flow through activated carbon dechlorinated tap water for 2 days (11/28-30/00). Light was available for 16 hours per day and the temperature was kept at 21-22°C. Fish were fed frozen brine shrimp twice daily. After the acclimation period fish were divided among exposures.

Effluent Location - Polishing Wetland

- In fall of 1992 the City of Denton, Texas constructed a half-acre experimental treatment wetland at the Pecan Creek Water Reclamation Facility. The wetland measured approximately 46×46 meters with three earthen berms separating it into four lanes. Lane depth varied from a few cm near the inflow to 0.6 meters at the outflow.
- The wetland received dechlorinated final wastewater effluent and held an estimated maximum volume of 570,000L (~150,000 gallons). The effluent diverted to the wetland comes from the Pecan Creek Water Reclamation Facility, a 15 million gallon per day treatment facility that utilizes an activated sludge treatment system. The wetland was isolated from groundwater by a layer of clay. The inflow and retention time are adjustable.

Figure 1

Locations of where fish were deployed Site 1 and Site 3 where three cages with 20 fish in each were deployed for 3 weeks.

Fish Secondary Sex Characteristics and Fitness Endpoints

- After exposure, fish were taken to the laboratory and sacrificed, length (cm), weight (g), testes weight (g), liver weight (g), hematocrit (% packed cells) and secondary sexual characteristics (SSC) including number of tubercles, fatpad thickness and stripe density were recorded.
- Blood plasma was isolated and VTG quantified at the University of Florida Biomarkers/Protein Chemistry Core Facility as described below.
- Condition factor (K; weight 105 /length3)[13] and hematocrit (packed blood cell column height / total blood column height 100) were calculated for individual fish to assess fish health.
- Gonadal-Somatic Index (GSI; testes weight/somatic weight 100) and Hepatic-Somatic Index (HSI; liver weight/total body weight 100) were calculated to assess the physiological effects of estrogenic exposure.
- Fatpad thickness and stripe density were recorded semi-quantitatively using a rating system of 0=none, 1=small, and 2=moderate, and 3=strong display.

Protocol For Fathead Minnow Liver Sample Field Collection and Processing

1. Ten Fish were collected at each time interval, 0, 1, 2, 4, 7, 14, and 21-days after deployment.
2. At each time interval livers were necropsied from each individual after blood was taken for Vg Plasma analyses, scoring of fish endpoints (SSC, GSI, HSI etc.)
3. Livers were placed in label tubes containing 1.0 mL of RNALater®.
4. Tubes were then placed in 4°C refrigeration for 24 hours storage following sacrifice, then cover tube rack with cardboard strip and tape securely and ship tubes with ice blocks (plus a copy of the ID data sheet) in a styrofoam container.
5. At the end of the study all tubes were shipped back to Cincinnati for Vg gene expression analyses.

Analysis of vitellogenin gene transcription

- Isolation of total RNA. Total RNA was isolated from individual adult livers by the standard guanidinium isothiocyanate method [14]
- The RNA samples were then evaluated for purity and spectrophotometrically quantified with a Shimadzu UV-1601PC® (Shimadzu Scientific Instruments, Columbia, MD, USA).
- Total RNA was subsequently analyzed for structural integrity of 28S and 18S rRNA species by electrophoresis in formaldehyde/agarose (1X MOPS [3-(N-Morpholino) propanesulfonic acid]) gels.
- Prior to RT-PCR analysis, the RNA samples were diluted to a concentration of 1 µg/ml in diethyl pyrocarbonate-treated water.

Thermal amplification (RT-PCR) [18 & 19]

Gene-specific oligonucleotides were designed from the fathead minnow vitellogenin precursor mRNA sequence using Oligo Primer Analysis Software®.

- First strand cDNA syntheses were according to manufacturer's recommendation (Applied Biosystems): 1 µg total RNA, 5 mM $MgCl_2$, 1 mM deoxy(Guanosine, Adenosine, Thymidine, Cytidine) triphosphates [d(G,A,T,C)TP], 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 20 U RNase inhibitor, 2.5 mM random hexamers, and 2.5 U Moloney Murine Leukemia Virus (MuLV) reverse transcriptase.
- All components, except individual sample RNAs and reverse transcriptase, were combined into a master mix and aliquoted into individual thin-walled reaction tubes.
- A component mix for the secondary *multiplex* PCR reaction was added to the 20 m reverse transcription reaction for a final PCR reaction volume of 50 µl.
- This secondary master mix contained 21.5 µl of PCR-grade water, 5 µl of 10X Advantage 2® PCR buffer, 1 µl of 50X Advantage2® DNA polymerase, 150 nM of each *Vg* oligonucleotide primer, and 0.5 µl of 7:3 ratio of Competimer®/18S (Ambion Inc., Austin, TX) oligonucleotides per reaction.
- Inclusion of oligonucleotide primers, specific for 18S rRNA, permitted relative quantification of vitellogenin gene expression.

Analysis of vitellogenin Plasma Levels

- After exposure, fish were sacrificed and blood samples were collected. Blood samples were taken by severing the caudal peduncle with a razor and collecting blood with a heparinized hematocrit tube. Blood samples in hematocrit tubes were centrifuged at 3,000 RPM in a hematocrit centrifuge for 3 minutes. After centrifugation, the eluted plasma was removed from the hematocrit tubes with a Hamilton syringe and injected into aprotinin plated 1.5 ml Eppendorf tubes.
- The plasma samples were immediately placed on dry ice and prepared for shipment to the University of Florida Protein Chemistry Research Laboratory for plasma VTG quantification.
- Eppendorf tubes were plated with a 10 ul diluted working solution of 0.9% NaCl, 0.9% benzyl alcohol with 3 mg/ml aprotinin (10X solution). The working solution was prepared by diluting 10 ul of the 3 mg/ml solution to 1 ml with distilled water. Eppendorf tubes containing 10 ul of the working solution were then vortexed to coat the tubes and then allowed to dry. Eppendorf tubes were refrigerated after drying until the plasma was added.
- Collected plasma was quantified for VTG using direct enzyme linked immunosorbant assays (ELISA) as decribed by Denslow et. al. [15]. The monoclonal antibody 2D3 used for this assay was made specifically against carp VTG, however, it crossreacts with fathead minnow VTG. The direct ELISA required plating a series of VTG standards along with unknown sample VTG on microwell plates.
- Following the initial anti-VTG monoclonal antibody 2D3 binding, biotinylated goat anti-mouse IgG polyclonal antibody and strepavidin conjugated alkaline phosphatase were used consecutively.
- The bound enzyme then converted the substrate mixture of p-nitro blue tetrazolium/5-bromo-4-chloro-indoyl phosphate into a color product that absorbs light at 405nm. By extrapolating the unknown absorbance unit against the standard curve with the known VTG concentration, VTG concentration was calculated [15].
- To obtain concentrations within the standard curve a series of sample dilutions were performed. The samples were first diluted from 1 to 100 and subjected to direct ELISA with a detection limit of 0.5 ug/ml. Samples with higher concentrations than the standard curve range were subjected to further dilutions of 1:10,000, 1:100,000 or 1:1,000,000 and direct ELISA was performed again.
- Those samples with lower concentrations were repeated with a competitive ELISA at a dilution of 1:20 and 1:50. Competition ELISA is currently more sensitive than the direct ELISA with a minimum detection limit of 0.2 ug/ml of plasma (personal communication Marjorie Chow).

Analytical Methods for Estrogens (16,17)

- 1L water filtered through Empore SDB-XC extraction disks
- Disks eluted with two 15 mL aliquots of methanol
- 1-2 g Hot Sodium sulfate added
- Samples were taken to dryness under nitrogen
- Samples were derivitized with MSTFA/TMSI/DTE (1000:2:2 mixture)
- Analyzed via a Hewlett Packard 6890 Series GC with a Hewlett Packard 5973 Mass Selective Detector J&W DB-5MS capillary column (30 m x 250 um x 0.25um)
- Seletive ion monitoring with ions 425, 342, and 416 utilized for E2, estrone and EE2

Detection limits were 1 ng/L for all estrogens analyzed

RESULTS

Table 1 Chemistry Results of grabs samples collected at Site 1 and 3 on Days 1, 7 and 21.

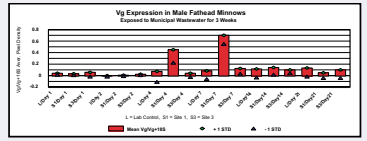
Site	Day	Equilenin ng/L	Estradiol ng/L	Estrone ng/L	Ethynylestradiol ng/L
1	1	nd.	5	5	25
1	7	n.d.	4	5	n.d.
1	21	n.d.	3	8	n.d.
3	1	n.d.	n.d.	9	n.d.
3	7	n.d.	n.d.	3	n.d.
3	21	n.d.	n.d.	n.d.	n.d.

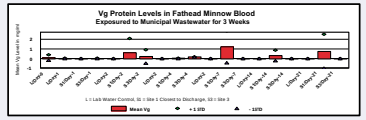
Table 2 General Water Quality Characteristics Over the 3 Week Exposure Period

Day	Date	Site	D.O.	Specific Cond	pH	Temp	NH4	Turb	TDS	Chlorine
			mg/L	Umhos/cm		C	mg/L	NTU	mg/L	mg/L
Day 0	11/30/00	Site 1	6	773	7	19.2				
		Site 3	4	785	7.2	7.6				
		Lab	9	420	8	23				
Day 1	12/01/00	Site 1	6.6	708	7.1	19.9	0.5	10.8	539.6	329.3
		Site 3	5.4	581	7.3	11.4	0.3	0.03	548	479.3
		Lab								
Day 2	12/02/00	Site 1	6.2	768	7.2	18				
		Site 3	8.64	791	7.2	6.8				
		Lab								
Day 4	12/04/00	Site 1	5.92	771	7.2	18.5				
		Site 3	6.95	774	7.5	7.8				
		Lab								
Day 7	12/07/00	Site 1	7.22	809.5	6.94	16.68	0.56	12.4	517.3	445.5
		Site 3	10.4	726.1	7.05	4.04	0.39	9.3	463.8	894.7
		Lab								
Day 14	12/14/00	Site 1	6.5	690	7.7	17	0.5	14.1	443.3	505.2
		Site 3	10.3	710	7.9	6.8	0.41	14.3	464.9	836
		Lab								
Day 21	12/21/00	Site 1	6.79	786.5	6.6	15.58	0.9	24.2	502.8	58.32*
		Site 3	7.15	778.1	6.89	4.02	0.84	9.2	498.6	71.65*

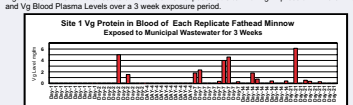
*Suspect value doesn't correspond with 7 months of previous chlorine measures with this meter.

Figures 2 and 3. Depict the pattern in Vitellogenin Gene Expression and Blood Plasma Vitellogenin Protein over 3 weeks





Figures 4 and 5 Show the results of individual fish exposed at Site 1 for Vg Expression in Male Livers and Vg Blood Plasma Levels over a 3 week exposure period.



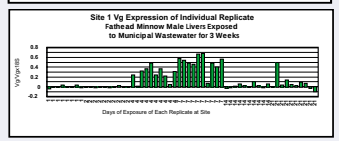


Figure 6 Shows the change in Tubercal Numbers in Male Fathead Minnow at Days 1, 7. 14 and 21 Days

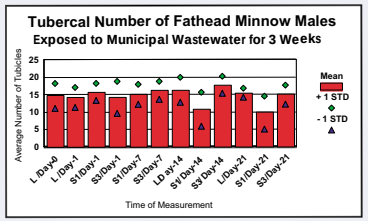


Figure 7 Shows the change in Stripe Condition of Male Fathead Minnow at Days 1, 7 14 and 21 Days

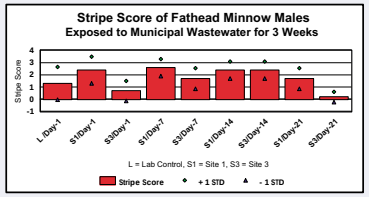


Figure 8 Shows the change in Gonadal-Somato-Index in Male Fathead Minnow at Days 1, 7, 14 and 21 Days

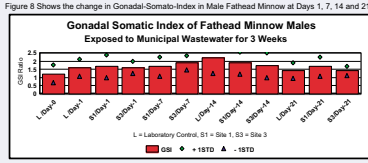


Figure 9 Shows the change in Heptic-Somato-Index in Male Fathead Minnow at Days 1, 7, 14 and 21 Days

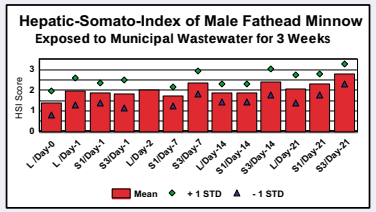
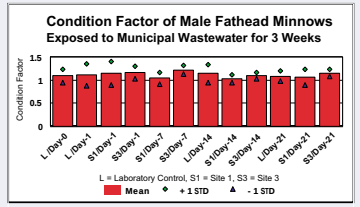


Figure 10 Shows the Condition Factor Levels in Male Fathead Minnows at Days 1, 7, 14 and 21 Days



CONCLUSIONS

Vg Gene Expression - Figure 2

1. Detectable Vg gene expression was first observed at day 4 at Site 1.
2. Vg gene expression peaked at Day 7 at Site 1 and then decreased to baseline through Day 21.
3. Site 3 showed no Vg gene expression greater than the lab control.

Vg Protein Plasma Levels - Figures 3&4 and Replicate Vg Gene Expression Figure 5

1. Vg Protein levels were only detected at Site 1 on days 2, 7, and 21. However, variance was too great to say that these differences were significant and when looking at individual replicate results (Figure 4) only day 7 shows Vg protein levels in a majority of the replicate analyses.
2. Vg gene expression measured in individual fish was less variable than measures of Vg protein in individual fish - Figures 4&5.

Table 1

1. Only Ethynylestradiol was found at a significant level on day 1. However, our experience with laboratory exposures at at similar levels (20 ng/L) indicate that little of the Ethynylestradiol measured is bioavailable because we should have measured high levels of gene expression on days 1 and 2 instead of on days 4 and 7. It is also possible that the Vg gene expression measured on days 4 and 7 were due to some unmeasured estrogenic compound.

Figures 6 - 10 Secondary sex characteristics and fitness measures

1. Tubercal number was the only indicator that showed an expected effect due to long term exposures to estrogenic compounds, i.e. tubercal number decreased after 2 weeks of exposure at Site 1. Fatpads were significantly less with increased duration of exposure to site 1.
2. HSI was significantly increased at both site 1 and site 3.

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